SERUM IL17 AND IL4R rs1805010 GENOTYPES: RELATIONSHIP WITH RHEUMATOID ARTHRITIS DISEASE ACTIVITY IN EGYPTIAN PATIENTS

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ABSTRACT

Rheumatoid arthritis(RA) is characterized by the presence of a relative state of imbalance between pro- and anti-inflammatory cytokines such as Interleukin(IL)17 and IL4, respectively. IL4 is supposed to regulate production of IL17 from T-helper (Th)17 cells. However, this regulatory function might be affected by singlenucleotide polymorphism (SNP) of IL4 receptor (*IL4R)* gene, rs1805010. The current study aimed to assess serum IL17 level in Egyptian patients with RA according to*IL4R*rs1805010 genotypes,and to detect possibleassociations betweenIL17/ *IL4R* genotypesand clinical status, disease activity as well as effect of treatment. Serum IL17 was assessed by ELISA, and qPCR was used to determine the genotypes of *IL4R* SNP rs1805010. Serum IL17 was significantly increased in patients' samples as compared to controls. According to *IL4R* genotypes, patients with AG and GG genotypes showed significantly higher IL17 levels than control subjects with corresponding genotypes. Within RA group, significantly higher IL17 were found in GG carriers compared to those with AA genotype. The G allele was significantly associated with higherythrocyte sedimentation rate(ESR), increased disease activity score in 28 joints

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(DAS28), highLarsen score and seropositive rheumatoid factor (RF) as well as C-reactive protein (CRP).Patients with AG and GG genotypes demonstrated significant positive correlations between serum IL17 and DAS28.Meanwhile, serum IL17 levels and Larsen score had significant positive correlation only in GG patients.The use of different treatment regimens did not affect serum IL17 levels significantly in various genotypes. In conclusion, IL17 may be implicated in the pathogenesis of RA, being associated with a higher disease activity parameters, however, its action may be potentiated due to loss of the functional *IL4RA* allele (rs1805010), particularly in carriers ofthe GG genotype. Furthermore, determining the genetic variants of *IL4R* rs1805010 may be promising for identification of patients at risk worse prognosis.

***Key words*:**Autoimmune disease; pro-inflammatory cytokines;*IL4R* genotypes.

INTRODUCTION

Rheumatoid arthritis is a systemic inflammatory disorder of chronic nature, associated with destructive synovitis, progressive cartilage and bone damage. The wide prevalence of RA, about 0.5–1% of the population worldwide, stratifies it as one of the most common autoimmune diseases. Though its exact etiology is still unknown,knowledge related to its pathogenesis is expanding and currently is thought to be driven by complex interactions of cells, like T cells, macrophages and fibroblasts***(Kugyelka et al., 2016).***

Previously, CD4+ lymphocytes were thought to comprise two discrete effector cells, the Th1 and Th2 subsets which are responsible for secretion of either interferon (IFN)-γ or IL4, respectively. In 2005, a third T helper cell subset,producing IL-17, was reported and called Th17 cells***(Harrington et al., 2005 and Park et al., 2005).***Before Th17subset was discovered, IL-17 was suggested to originate from Th1 cells, however, IL-12 was found to inducetransformation ofhuman Th17 cells into Th1 cells ***(Boniface et al., 2010)***.

Former studies detected Th17 cells close to osteoclasts in joints of RA patients. They also suggested thatIL17promotes osteoclast differentiation, hence aggravates joint destruction and bone resorption during RA. In addition, local and systemic production of IL17 was detected during initiation of RA in animal models. ***(Kugyelka et al., 2016).***Interestingly, in vitro studies suggested that IFN-γ and IL4,

produced by other Th cell subsets, can suppress Th17 cell development anddownregulateIL17 production*(Astry et al., 2011 and Cooney et al., 2011).*

The role of IL4 in counteracting RA inflammation is affected by functional SNPs in *IL4R(Prots et al., 2006)*.Replacementof isoleucine (I) byvaline (V) in amino acid chain of IL4R is controlled by SNP(rs1805010) in *IL4R*coding region. Accordingly, the function ofIL4R is modulated *(Wallis et al., 2011).*

Understanding the interaction between genetic and other molecular markers, such as *IL4R* rs1805010 and IL17, may represent a powerful tool for predicting diagnosis, prognosis, improving the treatment strategies, and achieving better outcome of RA disease *(Krabben et al., 2013 and Wallis et al., 2011).*From this point, the current study aimed to explore the differences in serum IL17 levels according to *IL4R* genotypes (rs1805010) in Egyptian patients with established RA. Furthermore, toelucidate theirpossible association with RAclinical manifestations and treatment effect.

MATERIALS & METHODS

The current study included 170 RA patients who were diagnosed according to 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria(Aletaha et al., 2010). They were selected during regular follow-up at the outpatients' clinic and the inpatients' unit of the Rheumatology and Rehabilitation Department, Benha University Hospitals. Additional 133, age and sex matched, apparently healthy volunteers were recruited as a control group.We excluded patients who were less than 18 years old and thosepresented with overlap or association with any other rheumatic or autoimmune diseasese.gosteoarthritis, psoriatic and lupus arthritis. Control subjects with seropositive RF, CRP, ESR oranti-cyclic citrullinated peptide antibody(Anti-CCP) results were also excluded.The study scheme was approved by the ethical committee of Faculty of Medicine, Benha University andinformed consents were obtained from all participantsbefore enrollment.

Upon enrollment, all patients were subjected to full history taking, complete physical examination, visual analogue scale (VAS) and disease activity assessmentusing the Disease Activity Score in 28 joints (DAS28)*(Aletaha et al., 2005)*. Plain radiographs were obtained

and the Larsen Grading Scale*(Larsen 1995)* was used to determine the extent of radiographic changes progression due toRA. The scale scored the extent of joint space narrowing, bony outlines, and degree of erosions. A mean score was calculated separately for the hands, wrists, and feet. The scores of all 32 areas wasaveraged together.

Blood sampling:venous blood was withdrawn from each subject, separated into two parts: the first part was collected into EDTA vacutainers to be used for erythrocyte sedimentation rate assay (Westergreen method) and genotyping assay of *IL4R* at SNP rs1805010. The secondpart was used to separate serum forRheumatoid Factor*(Dorner et al., 1987),*C-reactive protein *(Dixon et al., 1984)*, anti-cyclic citrullinated peptide antibody *(van Gaalen et al., 2005)* assays and the remaining serum was stored at -20°C for later assessment of IL17 level.

Assessment IL17 level: was performed by Infinite F50 absorbance reader (Tecan, Austria) using commercial ELISA assays (Biosource, Camarillo, USA) according to the manufacturer's instructions*(Conti et al., 2012)*

***IL4R* Genotyping:** whole blood samples were used for DNA extraction using the QIAamp DNA Blood Mini Kit (Qiagen, Germany) following the manufacturer's protocol. DNA concentration was determined using Nanodrop 2000c spectrophotometer (Thermo Scientific, USA). Genotyping of*IL4R, rs*1805010 was performed using TaqMan® Predesigned SNP Genotyping Assay (Applied Biosystems, USA) and TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems, USA) according to the manufacturer's instructions.Genotyping assays were performed and analyzed by StepOne real time PCR system (Applied Biosystems, USA).

Statistical analysis: data were summarized in terms of mean ±SD for quantitative data and frequencies and percentages for qualitative data. Comparisons between the different studied groups were carried out using the Chi-square test (χ^2), Fisher's Exact Test (FET) and the test of proportion (Z) to compare proportions as appropriate. The Student t-test (t) and Mann-Whiteny test (z) were used to detect differences between two normally and non-normally distributed variables respectively, while the ANOVA (F) and Kruskal Wallis test (χ^2) were used to compare more than two quantitative data. Genotype frequencies in RA cases and controls were tested for Hardy–Weinberg equilibrium, and significanceof any deviation between the observed and expected frequencies was tested using Chi- square test.

A p-value <0.05 was considered statistically significant. The statistical analysis was conducted using STATA version 11.2 (STATA corporation, College Station, Texas).

RESULTS

The current study included 170 RA patients (147 females, 86.47% and 23 males, 13.53%). Their ages ranged between 30 and 58 years with a mean of 41.49±5.57 years. Other clinical, laboratory and radiologic characteristics of the study groups are shown in table (1)

Table (1): Baseline characteristics of the study groups

Parameter		RA patients (n=170)	Controls (n=133)
Disease duration (years)	Mean±SD	3.14±2.35	NA
Duration of morning stiffness (minutes)		66.56±48.82	NA
Tender joint count		4.52±3.39	NA
Swollen joint count		3.85±2.71	NA
VAS		5.82±9.3	NA
DAS28(disease activity)		4.27±1.16	NA
ESR (mm/1st hour)		49.25± 22.07	11.14± 4.15
RF positive (%)	n (%)	154 (90.59%)	0 (0%)
CRP positive (%)		129 (75.88%)	0 (0%)
anti-CCP positive (%)		144 (84.70%)	0 (0%)
Larsen scale Grade 1 (%) Grade 2 (%) Grade 3 (%) Grade 5 (%)		63 (37.06%) 89 (52.35%) 16 (9.41%) 2 (1.18%)	NA

n.: number**, NA:** not applicable**, VAS:** visual analogue scale**, DAS28:** Disease Activity Score in 28 joints.

IL4R genotyping (SNP rs1805010) results were in accordance with Hardy-Weinberg equilibrium. There were90 patients of AG genotype (52.94%), while each of the homo-genotypes (AA or GG) was found in 40patients (23.53% each). Regarding the control group, the frequencies were 63(47.37%),37 (27.82%) and 33(24.81%) for the AG, AA and GG genotypes, respectively. Notably, differences in

genotype frequencydistribution between the controls and RA patients were insignificant (p>0.05).

Regarding serum level of IL17, sera of RA patients had significantly higher levels as compared to serum samples of the controls (p< 0.05). Based on genotype classification of both studied groups, IL17 was significantly increased only in AG and GG patients compared to healthy subjects with corresponding genotypes. Importantly, within RA patients, those with GG genotype had significantly elevated serum IL 17 levels as compared to AA patients (p< 0.05). Figure (1).
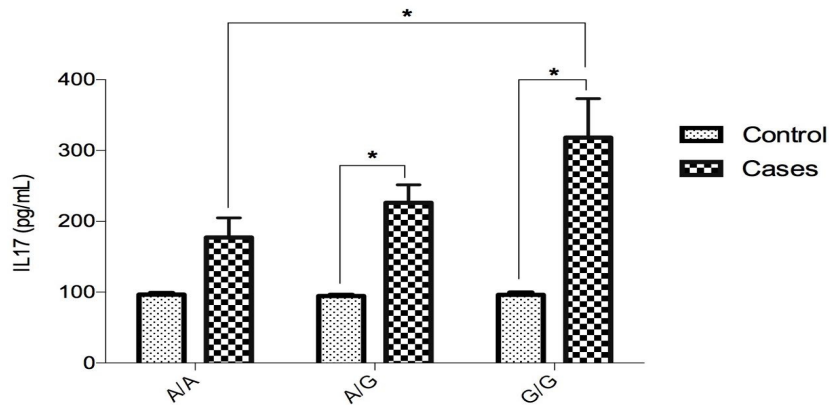
*significant difference p<0.05

Figure (1): comparison of serum IL17 levels between different genotypes in RA patients and control groups

Within RA patients' group, comparison of various genotypesas regard mean values of DAS28 scale and ESR revealed that they were significant higherin AG patients when compared to those with AA genotype, and in GG patientscompared to AA or AG subgroups, as well (all p<0.05).In addition, the current results showed significant differences in frequency distribution of DAS28 grades with GG patients having the highest frequency of the high DAS28 grade (p<0.05).

Moreover, patients who had GG genotype showed significantly higher frequencies of positive RF and CRP results, as compared to other genotypes.Higher grades of Larsen scale presented with significant association to GG genotype as well (p<0.05). Table (2).

Table 2:Association of rs1805010 genotypes with clinical and laboratory features of RA

Variable	Mean ±SD		
	Homo A/A (n=40)	Hetero A/G (n=90)	Homo G/G (n=40)
DAS28	3.28±1.14	4.30±0.87*	5.18±0.99*·†
Remission (<2.6)	21 (52.5%)	5 (5. 6%)	2 (5%)
Low (2.6 to <3.2)	6 (15%)	17 (18.9%)	2 (5%)
Moderate (3.2 to5.1)	10 (25%)	58 (64.4%)	7 (17.5%)
High (>5.1)	3 (7.5%)	10 (11.1%)	29 (72.5%)
ESR	33.5±17.8	48.9±19.11*	65.73±20.7*·†
RF			***
Negative	9 (22.5%)	5 (5.6%)	2 (5%)
Positive	31 (77.5%)	85 (94.4%)	38 (95%)
CRP			***
Negative	18 (45%)	19 (21.1%)	4 (10%)
Positive	22 (55%)	71 (78.9%)	36 (90%)
Larsen scale			***
Grade 1	5 (12.5%)	42 (46.7%)	16 (40%)
Grade 2	26 (65%)	41 (45.6%)	22 (55%)
Grade 3	9 (22.5%)	7 (7.7%)	0 (0.0%)
Grade 5	0 (0.0%)	0 (0.0%)	2 (5%)
Anti-CCP			
Negative	5 (12.5%)	15 (16.7%)	6 (15%)
Positive	35 (87.5%)	75 (83.3%)	34 (85%)

* Significant differences compared to Homo A/A; † significant differences compared to Hetero A/G,
***significant difference between studied genotypes, p<0.05: significant

In addition, the present study revealed that in patients with AG, GG genotypes, serum IL17 levels were significantly positively correlated to DAS28. However, IL17 serum levels were positively correlated to Larsen scale with significant difference only in patients with GG genotype.Table (3)

Table 3:Correlation coefficient (ρ) between DAS28, Larsen score and serum IL17 levels in various genotypes of RA patients

		DAS28	Larsen scale
Serum IL17	**A/A**	0.243	0.145
	A/G	0.536*	0.089
	G/G	0.439*	0.668*

ρ: Spearman correlation coefficient (rho), *** p**<0.05 significant
Finally,RA patients included in this study were using different combination of disease-modifying anti-rheumatic drugs (DMARDs). within each *IL4R* genotype, the use of different treatment regimen did not affect serum IL17 levels significantly(p>0.05 all).

DISCUSSION

Previous studies suggested a key role for IL17 in the pathogenesis of RA and pointed to the association between *IL4R* SNP rs1805010, and RA joint destruction *(Krabben et al., 2013)*. This study was conducted on Egyptian RA patients to assess the differences in serum IL17 levelsbased on genotypes of *IL4R* SNP rs1805010,and to elucidate their possible association with RA clinical manifestations and treatment effect. To our knowledge, the current work is the first study in Egyptian RA patients to and focus on the relationship between IL4R genotypes and serum IL17, providing more insight on mechanism of action of IL4R with different genotypes.

The role of IL4R genotypes in RA susceptibility in Egyptian patients was previously reported with contradictory results (**Hussein et al., 2012 and Mohamed etal., 2015).**The present data showed non-significant differencesin genotypes' distribution between RA patients and controls. This result supportsthe previous reports that*IL4R*SNP (rs1805010) might not be relevant as a risk factor for RA disease*(Burgos et al., 2010 and Hussein et al., 2012)*. However, this observation was in contrast to that of **Mohamed etal., 2015.** The different samople sizes and use of different technicques for detection of IL4R genotypes may explain such controversy.

Studying clinical activity parameters in different genotypes, results of the current study demonstrated a significant association between rs1805010 G allele from one side and the higher DAS28, ESR values on the other side. Moreover, patients who had GG genotype presented with significantly higher frequencies of seropositive RF, CRP and higher grades of Larsen scale. In this regard, our results were similar to what was previously reported. After follow up for one year, **Leipe and colleagues, 2014** demonstrated that carriers of G variant had significantly higher DAS28 scores than healthy controls.As well, an Egyptian study found that RF seropositivity and bone erosions associated significantly withG allele of *IL4R(Hussein et al., 2012)*. Other reports suggested that carrying G allele was predictive of more severe erosive disease *(Leipe et al., 2014 and Prots et al., 2006)*. Additional evidence was provided by another research group as they stated that moderate DAS scores was found in AG patients while GG patients showed higher scores. They added that though their observation was statistically insignificant, yet it reflects higher disease activity in GG genotype of rs1805010 *(Wallis et al., 2011)*.

However, one study in African American patients with RA, failed to detect any significant association between genotypes of rs1805010 and RF status, Anti-CCP or radiographic erosions. Such discrepancy may be attributed to different studied races, patients' characteristics and effect of treatment.

IL4 has been shown to be highly protective cytokine in RA due to its ability to suppress inflammation and joint destruction through many mechanisms. It inhibits production of vascular endothelial growth factor thus possesses antiangiogenic effect, inhibits osteoclastic activity and downregulatestissue metalloproteinase-1 expression and so decreases cartilage destruction*(Wallis et al., 2011)*.Interestingly, binding of IL4 to its receptor activates signal transducer and activator of transcription-6 (STAT6)which upregulates a wide range of genes necessary for Th2differentiation of CD4 T cells.In turn, enhanced differentiation to Th2 cells allows further increase of its product; IL4.*(Prots et al., 2006)*..

Though *IL4R rs1805010* is located near IL4- binding site, prior reports suggested that its effect is mediated through modulating the action of STAT6, rather than changing the affinity for IL4. It has been shown that IL4R-A allele confers protectionin RA patients, through prolonged STAT6 activation, thus increased IL4 production, while the G variant is associated with higher disease activity and rapidly developing erosions due to diminished STAT6 activation and decreased IL4 production *(Hussein et al., 2012; Leipe et al., 2014 and Prots et al., 2006)* In addition, assessment of serum IL17 revealed significantly higher levels in RApatients compared withcontrol subjects,which is consistent with the results of previous studies in earlyas well as in established RA. They attributed these results to expansion and enhanced activity of Th17 subset in RA patients compared to healthy individuals*(Al-Saadany et al., 2016; Edavalath et al., 2016 and Pavlovic et al., 2014)*or osteoarthritis patients*(Leipe et al., 2010 andWallis et al., 2011)*.RA was regarded as Th1-driven disease, but this concept has been changed after discovery of Th17 subset and its effector IL17, and currently is thought to be dependent mainly on Th17 cells.Through RA course, complex immune reactions lead to enhanced production ofmany proinflammatory cytokines including IL1β and IL6. Both cytokines have been shown to induce Th17 differentiation and to recruit Th17 to inflammation sites *(Kugyelka et al., 2016)*. A recent in vitro study demonstrated thatactivation of T-cell receptor and direct cell interaction with synoviocytes, were necessary for induction of IL17 secretionby Th17 cells in RA*(Noack et al., 2016)*.

Many mechanisms have been suggested to mediate the pathogenic effects of IL-17 during the progression of RA. At early stages of the disease, IL17 has been shown to stimulate the secretion of vascular endothelial growth factor by synovial fibroblasts, hence, increases neovascularization and angiogenesis. Moreover, IL17 enhances innate immunity through induction of expression of various inflammatory mediators and chemokines including complement system. Additionally, it acts as a chemotactic factor for monocytes and other inflammatory cells facilitating their infiltration into the

synovial tissues causing arthritis *(Astry et al., 2011 and Roeleveld and Koenders 2015)*Interestingly, stratification of the study groups according to their *IL4R* genotypes, demonstrated that the presence of G allele conferred significant increase of IL17 in patients as compared to corresponding controls. Furthermore,patients with GG genotype had the highest serum IL17 levels, followed by AG then AA genotypes. However, such differences were statistically significant onlyin between GG and AA patients.On the other hand, serum IL17 showed non-significant differences between the three genotypes in the control group. In line with these data,*Leipe and colleagues, 2014*demonstrated that presence of even one G allele was associated with significantly higher frequency of Th 17 and IL17 production in RA patients.

Furthermore, the present studydetected significant positive correlations between serum IL17 and DAS28 score in patients carrying AG and GG genotypes. However, only GG carriers showeda positive correlation with statistical significance between serum IL17 and Larsen scale.

Our results highlight the poor effect of *IL4R*G allele on RA progression, a process in which IL-17 seems to be a crucial player.Interestingly, IL4 has been shown todecrease autoimmune inflammation, bone and joint destruction not only through above mentioned mechanisms but also by suppression of Th17/IL17 axis and effects *(Cooney et al., 2011; Guenova et al., 2015; Leipe et al., 2014 and Wallis et al., 2011).*

The current findings might be explained by the reduced ability of IL4 to suppress Th17 cells in carriers of G allele. Amino acid substitution due to rs1805010 GG genotype, reduces STAT6 activation, hence weakens the downstream signals through IL4R. Thus, production of IL4 is significantly diminished and TH17/IL17 suppression is diminished as well, in such patients*(Cooney et al., 2011; Guenova et al., 2015; Leipe et al., 2014 and Wallis et al., 2011).*

Finally, there were non-significant differences in serum levels of IL17 between patients on different treatment regimens in all three genotypes. In this regard, the present study was limited because all included patients were using conventional DMARDs due to very high cost of other biologic drugs.

CONCLUSION : In conclusion,IL17 may play a role in progression of RA disease.However, clinical effects of IL17 become more evident due to loss of suppression by IL4 in patients with hypofunctionalIL4R of GG genotype. Determining *IL4R*rs1805010 genetic variants, may allow early identification of those at risk of high disease activityfor special therapeutic approachto reduce morbidity as possible. To establish the benefit of such patients from treatment depending on anti- IL17 rather than IL4 mechanisms, further longitudinal cohort studies are needed,with a larger sample size focusing on new biologic treatment strategies and their outcome.

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الملخص العربي

علاقة الانترلوكين ١٧في مصل الدم و النمط الجيني لجين مستقبل الانترلوكين ٤ (آر إس 1805010) بالنشاط المرضي في المصريين المصابين بالتهاب الروماتويد المفصلي

يتميز التهاب الروماتويدالمفصليبوجود حالة من عدم التوازن النسبي بين السيتوكينات المؤيدة وتلك المضادة للالتهابات مثل انترلوكين 17وانترلوكين ٤ على التوالي. ومن المفترض أن انترلوكين ٤يعمل على تنظيم إنتاجانترلوكين١٧ من خلاياتي المساعدة ١٧، ومع ذلك، فإن هذه الوظيفة التنظيمية يمكن أن تتأثر بالتعدد الجيني لأشكال لتغير الأحادي للنيوكليوتيدات (إس إن بي آر إس 1805010)لجين مستقبل الانترلوكين٤.

وتهدف الدراسة الحالية إلى تقييم مستوى الانترلوكين 17 في الدم في المرضى المصريين الذين يعانون من التهاب الروماتويد المفصلي وفقا للنمط الجيني لجين مستقبل الانترلوكين٤(إس أن آر إس 1805010) ، وكشف الصلة المحتملة بين الانترلوكين 17/النمط الجيني لجين مستقبل الانترلوكين٤ وكل من الحالة السريرية، النشاط المرضي وكذلك تأثير العلاج.

تم قياس مستوى الانترلوكين ١٧في مصل الدم بواسطة الإليزا، في حين استخدمت تقنية تفاعل البلمرة المتسلسل الكمي لتحديد الأنماط الجينية لجين مستقبل الانترلوكين٤(إس بي آر إس 1805010). وقد كشفت نتائج الدراسة عن زيادة ملحوظة ذات دلالة احصائية في عينات المرضى مقارنة بالمجموعة الضابطة.

وفقا للنمط الجيني لجين مستقبل الانترلوكين٤ ،لوحظ أن مستوى الانترلوكين ١٧في مصل الدم للمرضى الحاملين لنمطي اى جي و جي جي أعلى بصورة ذا ت دلالة إحصائية من الأشخاص في المجموعة الضابطة الحاملين لنفس الأنماط الوراثية.

ضمن مجموعة المرضبالتهاب الروماتويد المفصلي ، وجد أن مستوى الانترلوكين ١٧في مصل الدم للمرضى ذوو النمط الجيني جي جي مرتفع بفرق ذي دلالة إحصائية ، مقارنة مع حاملي النمط الجيني اى اى. كما لوحظ ارتباط الأليل جي معكل من ارتفاع معدل الترسيب، وزيادة مقياسالنشاط المرضي في ٢٨ مفصل، وارتفاع مقياس لارسن ومستوى عامل الروماتويد المفصليفي مصل الدم، وكذلك بروتين سي التفاعلي.

وقد أظهرت النتائج وجود علاقة طردية ذات دلالة إحصائية بين مستوى الانترلوكين ١٧في مصل الدم ومقياسالنشاط المرضي في ٢٨ مفصل في المرضى الحاملين للأنماط الجينية من نوعي اى جي و جي جي. وفي الوقت نفسه، كانت مستويات الانترلوكين ١٧في مصل الدممرتبطة ايجابيا بنتائج مقياس لارسن بدلالة إحصائية فقط في المرضى من النمط الجيني جي جي. ومن الجدير بالذكر ملاحظة أن استخدام نظم العلاج المختلفة لم تؤثرعلى مستويات الانترلوكين ١٧في مصل الدمبصورة ذات قيمة إحصائية في مختلف الأنماط الجينية.

ونستنتج من هذه الدراسة، أن الانترلوكين١٧ قد يعد عاملا مسببا في التهاب الروماتويد المفصلي ، كونه مرتبطا طرديا مع أعلى معايير النشاط المرضي، ومع ذلك،فإن تأثيره قد يزداد قوة نتيجةلفقدان وظيفية مستقبل الانترلوكين٤ لا سيما في حالة وجود الأليل جي جي (آر إس 1805010)،وبناءا على ماسبق، فإن تحديد النمط الجيني لجين مستقبل الانترلوكين٤ (آر إس 1805010)قد يكون واعدا لتحديد المرضى الأكثر عرضة للحالات المرضية الأسوأ.